Pituitary Tumor Transforming Gene 1 Induces Tumor Necrosis Factor-α Production from Keratinocytes: Implication for Involvement in the Pathophysiology of Psoriasis

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Proliferation and differentiation in the epidermis must be tightly regulated. This regulation is known to involve a range of transcription factors, including pituitary tumor transforming gene 1 (PTTG1), a ubiquitously distributed transcription factor that regulates keratinocyte proliferation and differentiation. Psoriasis is a common but refractory skin disorder, the pathophysiology of which is characterized by hyperproliferation and impaired differentiation in the epidermis. The present study was conducted to clarify the less well-known roles of PTTG1 in the pathophysiology of psoriasis, focusing on its relationship with tumor necrosis factor-α (TNF-α), which is a critical mediator of the disease. The levels of PTTG1 expression were increased in the psoriatic epidermis. Overexpression of PTTG1 resulted in the overproduction of TNF-α, and TNF-α itself had an inductive effect on PTTG1 expression, suggesting that their expression may involve autoinduction. Moreover, overexpression of PTTG1 involved augmented the expression of cyclin A and B1 proteins in both cultured keratinocytes and the psoriatic epidermis. Therefore, enhanced expression of PTTG1 in the psoriatic epidermis may result in aberrant regulation of the cell cycle and impaired differentiation via the interplay between PTTG1 and TNF-α.

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INTRODUCTION

Proper regulation of proliferation and differentiation in the epidermis is indispensable for maintenance of barrier function against the external environment (Proksch *et al.*, 2008). This regulation is known to involve a number of molecules, including transcription factors (Eckert *et al.*, 1997; Eckert *et al.*, 2011), the dysregulation of which results in skin disorders (Honeycutt *et al.*, 2004). Pituitary tumor transforming gene 1 (PTTG1), also known as securin, is a ubiquitously distributed transcription factor that has been shown to be involved in a number of cellular processes, including cell cycle control, organ development, angiogenesis, metabolism, cell transformation, and cell senescence (Pei and Melmed, 1997; Zou *et al.*, 1999; Tong and Eigler, 2009). Recently, we demonstrated that PTTG1 enhanced proliferation and suppressed differentiation of keratinocytes (Ishitsuka *et al.*, 2012), suggesting that it may have important roles in hyperproliferative disorders in the epidermis, such as psoriasis and skin cancers.

Psoriasis is a common but refractory skin disorder, the pathophysiology of which is characterized by hyperproliferation and impaired differentiation in the epidermis. Among a range of mediators involved in the pathogenesis of psoriasis (Proksch *et al.*, 2008; Nestle *et al.*, 2009), tumor necrosis factor-α (TNF-α) has been shown to have a critical role (Nickoloff *et al.*, 1991). Indeed, overexpression of TNF-α has been reported in the psoriatic epidermis (Kristensen *et al.*, 1993; Ettehadi *et al.*, 1994) in addition to the dermis (Lowes *et al.*, 2005; Clark and Kupper, 2006). Recent advances in biological therapy against such molecules (Langley, 2012) revealed that neutralization of TNF-α in psoriatic lesions restores normal epidermal differentiation (Kim *et al.*, 2011; Donetti *et al.*, 2012), suggesting important roles of TNF-α in the epidermal barrier function. Although the functions of TNF-α in immunity and inflammation have been elucidated (Aggarwal *et al.*, 2012), its roles in the epidermal barrier function are less clear. Moreover, as PTTG1 may have important roles in keratinocyte proliferation and differentiation (Ishitsuka *et al.*, 2012), it is important to clarify the relationship between PTTG1 and TNF-α in the epidermis and the regulatory machinery that may lead to a deeper understanding of the pathophysiology of psoriasis.

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Abbreviations: EGF, epidermal growth factor; FLG, filaggrin; K1, keratin 1; K10, keratin 10; mRNA, messenger RNA; NHK, normal human keratinocyte; NMK, neonatal mouse keratinocyte; PBS, phosphate-buffered saline; PTTG1, pituitary tumor transforming gene 1; RT–PCR, real-time PCR; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α

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The present study was performed to clarify the less well-known roles of PTTG1 in the pathophysiology of psoriasis and functions of TNF-α in keratinocyte proliferation and differentiation using cultured keratinocytes.

RESULTS

PTTG1 expression is enhanced in the psoriatic epidermis

As demonstrated in our recent study (Ishitsuka *et al.*, 2012), PTTG1 may have a critical role in keratinocyte proliferation. First, to confirm the impact of aberrantly expressed PTTG1 on keratinocyte proliferation, PTTG1 was constitutively overexpressed in normal human keratinocytes (NHKs) and WST1 proliferation assay was performed. NHK-overexpressing PTTG1 showed higher proliferation capacity than controls (Figure 1a). Next, to confirm the expression profile of PTTG1 in psoriatic epidermis, immunofluorescence and immunohistochemical staining for PTTG1 were performed in specimens obtained from patients with psoriasis ($n=12$) and normal controls ($n=6$), and its expression levels were compared among normal (Figure 1b), marginal ($n=10$) (Figure 1c), and lesional ($n=12$) (Figure 1d) areas along with the cell cycle marker Ki67 (Gerdes *et al.*, 1984), the initial differentiation marker keratin 10 (K10) (Roop *et al.*, 1983; Regnier *et al.*, 1986), and the terminal differentiation marker filaggrin (FLG) (Fisher *et al.*, 1987; Rothnagel *et al.*, 1987). In the normal controls and the marginal areas of the psoriatic epidermis, PTTG1 was expressed in the basal and peribasal keratinocytes, and was localized in the nucleus and cytoplasm (Figure 1b and c). Reflecting the cell cycle–dependent expression of PTTG1 (Ramos-Morales *et al.*, 2000), PTTG1 and Ki67 tended to be colocalized. There were no apparent aberrations in differentiation status as indicated by the expression patterns of K10 and FLG, in comparison with the normal controls (Figure 1b and c). On the other hand, PTTG1 expression was enhanced in the lesional areas of psoriatic epidermis; there were increased numbers of PTTG1-positive keratinocytes, and even keratinocytes above the peribasal layers showed strong immunoreactivity (Figure 1d). Similarly, the number of Ki67-positive keratinocytes was increased (Figure 1d). Irrespective of the lesional or marginal areas, the numbers of PTTG1- and Ki67-positive keratinocytes were significantly increased in the psoriatic epidermis compared with the normal controls (Figure 1e). On the other hand, the expression levels of K10 and FLG were significantly decreased in the lesional areas compared with the marginal areas and the normal controls (Figure 1d and e). Taken together, these findings suggested that the enhanced expression of PTTG1 is associated with the disease activity characterized by enhanced proliferation and impaired differentiation of the epidermis.

PTTG1 expression is induced by the EGFR-mediated proliferation signal in keratinocytes

Proliferation of epidermal keratinocytes is regulated by a variety of mediators represented by ligands of EGFR, including EGF and transforming growth factor-α (TGF-α; Cohen, 1962; Carpenter *et al.*, 1975; Massague, 1983). Overproduction of such ligands is one of the characteristic features of chronic inflammatory disorders, including psoriasis (Mascia *et al.*, 2003). To analyze their effects on keratinocyte proliferation and PTTG1 expression, neonatal mouse keratinocytes (NMKs) (Figure 2a and b) and NHKs (Figure 2c and d) were cultured and stimulated with different doses of EGF and TGF-α, respectively. First, the effects of EGFR ligands on keratinocyte proliferation were analyzed. As expected, the ligands enhanced keratinocyte proliferation as determined by WST1 proliferation assay (Figure 2a and c). Next, the effects of the EGFR ligands on transactivation of PTTG1 were analyzed by quantitative real-time (RT) PCR in the keratinocytes. The ligands induced PTTG1 messenger RNA (mRNA) expression in a dose-dependent manner (Figure 2b and d). These results suggested that PTTG1 is closely associated with proliferation capacity, and its expression is induced by the EGFR-mediated proliferation signal.

Overexpression of PTTG1 in keratinocytes induces the production of TNF-α

TNF-α is known to be overexpressed in the psoriatic epidermis and to have a key role in the pathophysiology of psoriasis (Kristensen *et al.*, 1993; Ettehadi *et al.*, 1994). Anti-TNF-α therapy can restore normal epidermal differentiation in patients with psoriasis (Kim *et al.*, 2011; Donetti *et al.*, 2012). On the other hand, the expression of PTTG1 in the lesional areas was enhanced, and overexpression of PTTG1 in cultured keratinocytes suppressed differentiation (Ishitsuka *et al.*, 2012). Therefore, it would be significant to analyze the effects of PTTG1 on TNF-α production in keratinocytes. First, the expression of TNF-α in cultured NHKs overexpressing PTTG1 was analyzed by immunoblotting and quantitative RT–PCR. The results indicated the upregulation of TNF-α in NHKs overexpressing PTTG1 (Figure 3a and b). Next, the concentration of TNF-α in the conditioned medium obtained from NHK cell cultures was analyzed by ELISA. The level of TNF-α secretion was increased by PTTG1 overexpression (Figure 3c). These results suggested that PTTG1 induces TNF-α production in keratinocytes.

PTTG1 expression is induced by TNF-α

Although TNF-α has been implicated in a range of cellular processes, including proliferation and differentiation via the activation of transcription factors (Aggarwal *et al.*, 2012), its effects on the regulation of PTTG1 expression remain unknown. Therefore, to clarify the effects of TNF-α on PTTG1 expression in keratinocytes, NMKs were cultured in medium containing different doses of TNF-α, and PTTG1 expression was analyzed by immunoblotting and quantitative RT–PCR. These analyses indicated that PTTG1 expression was induced by TNF-α in a dose-dependent manner (Figure 4a and b). Taken together with the observation that PTTG1 enhances the production of TNF-α, these findings strongly suggested a positive feedback cycle between PTTG1 and TNF-α.

TNF-α suppresses keratinocyte differentiation

Overexpression of PTTG1 suppressed keratinocyte differentiation (Ishitsuka *et al.*, 2012), and its expression was induced by

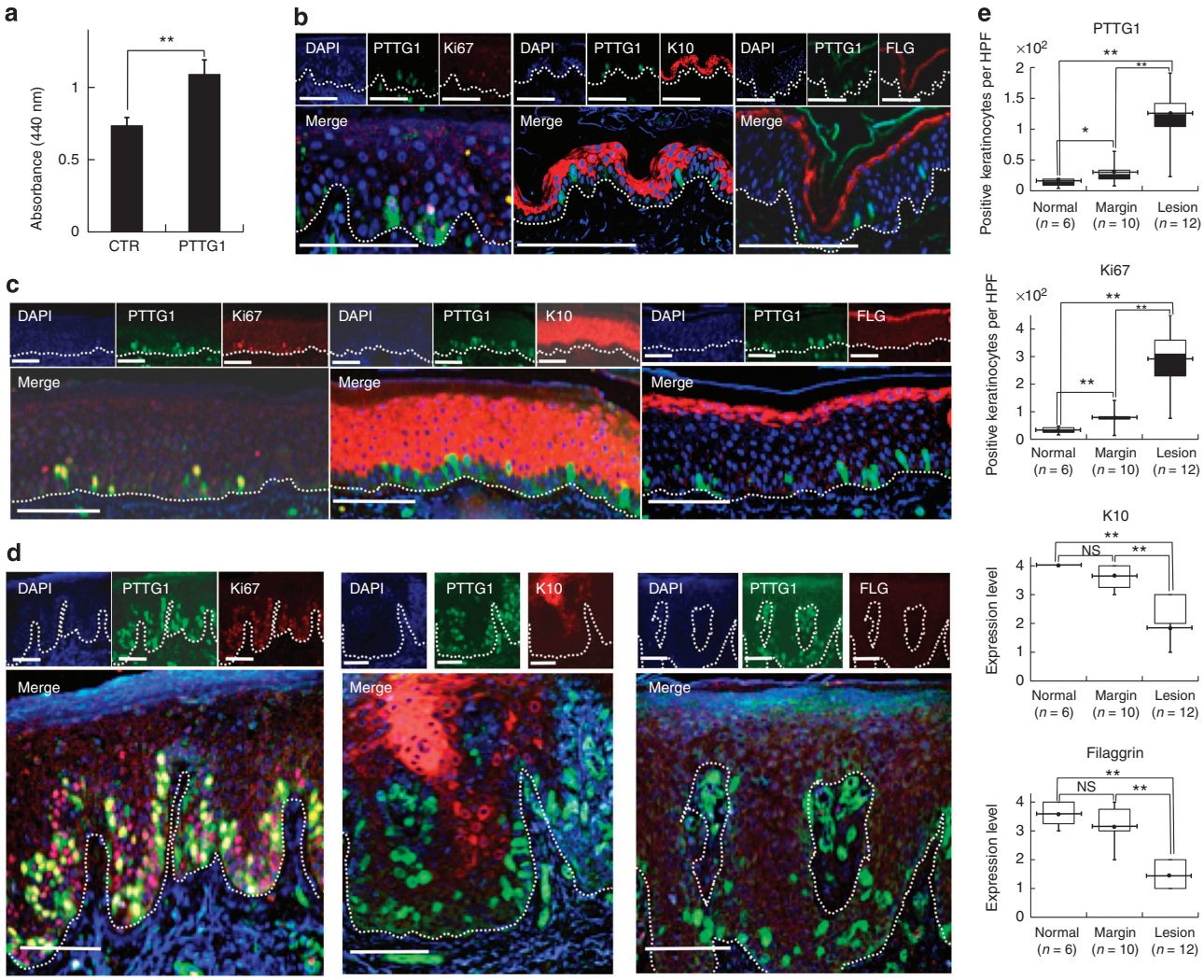

Figure 1. Pituitary tumor transforming gene 1 (PTTG1) expression is enhanced in the psoriatic epidermis. (**a**) PTTG1 was overexpressed in normal human keratinocytes (NHKs) via a retroviral vector and cultured in 96-well plates. Proliferation capacity was analyzed by the WST1 proliferation assay. The absorbance values at 440 nm was significantly increased in PTTG1-overexpressing NHKs compared with NHKs carrying a control vector (CTR). The experiments were repeated at least three times. (**b**–**d**) Sections of the samples obtained from patients with psoriasis and normal skin were incubated with an antibody against PTTG1 along with the proliferation marker Ki67, early differentiation marker keratin 10 (K10), and terminal differentiation marker filaggrin (FLG). Antibody binding was visualized using fluorescent antibodies with a confocal microscope. PTTG 1 was stained green and other immunogens were stained red. Representative images of (**b**) normal control and (**c**) marginal area of psoriatic epidermis. PTTG1 and Ki67 were located in the basal or peribasal layer. PTTG1 was expressed in the cytoplasm and the nucleus, and partially colocalized with Ki67 in the nucleus. K10 was expressed in the suprabasal layers and FLG was expressed in the granular layer. (**d**) Representative images of lesional area of the psoriatic epidermis. The numbers of keratinocytes positive for PTTG1 and Ki67 were increased. Stained keratinocytes were seen even in the middle layer of the epidermis. The expression levels of K10 and FLG were markedly decreased compared with the lesional area or the normal controls. White bars represent 100 mm. (**e**) Immunohistochemical staining was performed in samples obtained from patients with psoriasis (lesional area, $n=12$; marginal area, $n=10$; normal skin, $n=6$). The expression levels of PTTG1, Ki67, K10, and FLG were evaluated and compared among the groups. The numbers of PTTG1- and Ki67-positive keratinocytes were significantly increased in the lesional areas compared with the marginal areas and the normal controls. The positivity rates were higher in the marginal area than in the normal controls. The expression levels of K10 and FLG were significantly decreased in the lesional area compared with the marginal areas or normal controls, whereas there were no significant differences between the marginal areas and the normal controls. The bottom of the black boxes and top of the white boxes indicate the lower and upper quartiles, respectively. The borders of the black and white boxes represent the medians. The whiskers with black dots crossing the boxes represent the means. The lower and upper whiskers indicate the minimum and maximum values, respectively. *$P<0.05$ vs. control; **$P<0.01$ vs. control; DAPI, 4′6-diamidino-2-phenylindole; HPF, high-power field; NS, not significant.

TNF-α in NMKs. Therefore, to determine whether TNF-α itself alters keratinocyte differentiation, cultured NMKs (Figure 5a and b) and NHKs (Figure 5c) were induced to undergo differentiation by adding high concentrations of Ca^{2+} to the medium containing different doses of TNF-α. Immunoblotting and quantitative RT–PCR indicated dose-dependent decreases in expression levels of the early differentiation markers keratin 1 (K1), K10, and the terminal differentiation marker loricrin in NMKs (Figure 5a and b). On the other hand, the expression levels of K1, K10, FLG, and loricrin mRNA in NHKs were

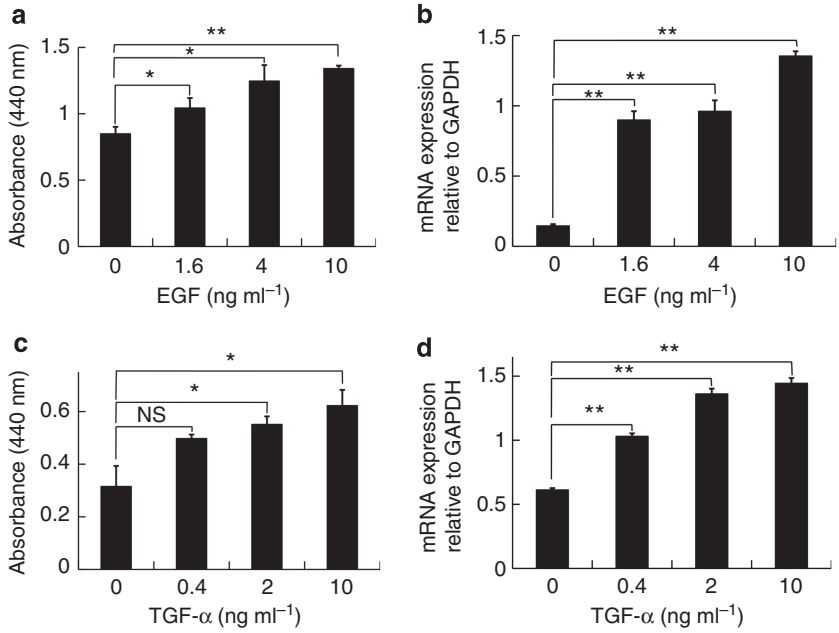

Figure 2. Pituitary tumor transforming gene 1 (PTTG1) expression is induced by the EGFR-mediated proliferation signal in keratinocytes. (**a**, **b**) Neonatal mouse keratinocytes (NMKs) were cultivated in growth medium containing different doses of EGF (0, 1.6, 4, and 10 ng ml^{-1}). (**c**, **d**) Normal human keratinocytes (NHKs) were cultivated in growth medium containing different doses of transforming growth factor-α (TGF-α; 0, 0.4, 2, and 10 ng ml^{-1}). The (**a**) NMKs and (**c**) NHKs were cultured in 96-well plates, and proliferation capacities of the keratinocytes were analyzed by the WST1 proliferation assay. Absorbance values at 440 nm were increased by EGF or TGF-α in a dose-dependent manner. Total messenger RNA (mRNA) was extracted from the (**b**) NMK and (**d**) NHK cultures, and the expression of PTTG1 was analyzed by quantitative real-time PCR. The expression levels of PTTG1 were increased in EGF and TGF-α dose-dependent manner. All data are means ± SEM from at least three independent experiments. *$P<0.05$ vs. control; **$P<0.01$ vs. control. NS, not significant.

decreased in the presence of TNF-α (Figure 5c). These results suggested that TNF-α suppresses keratinocyte differentiation.

PTTG1 overexpression in the psoriatic epidermis involves augmented expression of cyclin A and B1 proteins

A number of molecules related to the cell cycle are aberrantly expressed in the hyperproliferative psoriatic epidermis (Miracco *et al.*, 2000; Santos-Briz *et al.*, 2009; Abou EL-Ela *et al.*, 2010). Cyclins are known to regulate their binding partners, the cyclin-dependent kinases, and they drive the cell cycle transition at specific phases of the cell cycle (Quereda and Malumbres, 2009). Therefore, to analyze the effects of PTTG1 overexpression on cell cycle transition in the psoriatic epidermis, the expression profiles of cyclin A and B1 proteins were compared between cultured NHKs overexpressing PTTG1 and the psoriatic epidermis. In cultured NHKs, immunoblotting revealed that PTTG1 overexpression caused significant upregulation of cyclin A and B1 (Figure 6a). On the other hand, the lesional areas of psoriasis ($n=12$) showed significantly increased expression levels as indicated by the numbers of cyclin A– or cyclin B1–positive keratinocytes, compared with marginal areas ($n=10$) and normal controls ($n=6$), although there were no significant differences between the marginal areas and the normal controls (Figure 6c). These findings suggested that overexpression of PTTG1 in the psoriatic epidermis involves augmented cyclin A and B1 protein expression and may result in aberrant regulation of the cell cycle.

DISCUSSION

TNF-α is known to have a pivotal role in the pathophysiology of psoriasis (Nickoloff *et al.*, 1991), and therapeutic methods targeting this molecule are highly effective (Langley, 2012). In psoriatic lesions, not only macrophages (Clark and Kupper, 2006) and dendritic cells (Lowes *et al.*, 2005) located in the dermis but also epidermal keratinocytes are the sources of TNF-α (Kristensen *et al.*, 1993; Ettehadi *et al.*, 1994). We demonstrated that the production of TNF-α in keratinocytes was induced by PTTG1, the expression of which was increased by the EGFR-mediated proliferation signal and enhanced in the psoriatic epidermis associated with disease activity. The addition of TNF-α to NMK cell cultures increased the expression levels of PTTG1 and reduced the expression levels of differentiation markers in cultured NMKs and NHKs. Finally, PTTG1 overexpression involved the augmented cyclin A and B1 protein expression in both cultured NHKs and psoriatic epidermis.

PTTG1 had a positive effect on keratinocyte proliferation (Ishitsuka *et al.*, 2012), and PTTG1 expression was induced by the EGFR-mediated proliferation signal as demonstrated in earlier studies (Vlotides *et al.*, 2006; Vlotides *et al.*, 2008). Moreover, we confirmed increased secretion of EGF from cultured NHKs overexpressing PTTG1 by ELISA (data not shown). Therefore, the regulation of PTTG1 expression in keratinocytes may involve autoinduction depending on the proliferation status modulated by the EGFR-mediated signal, and this positive feedback cycle between PTTG1 and EGFR

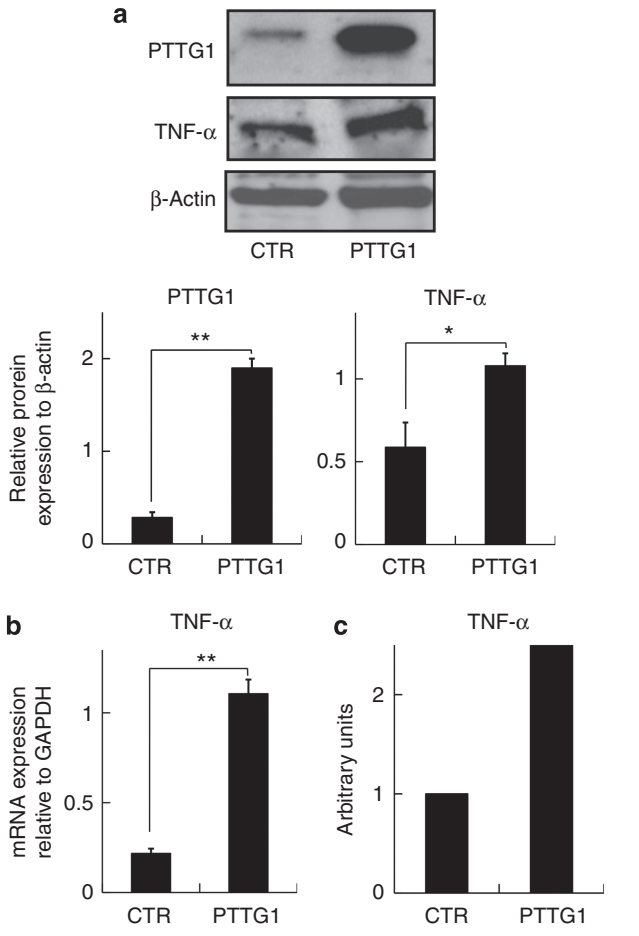

Figure 3. Overexpression of pituitary tumor transforming gene 1 (PTTG1) in keratinocytes induces the production of transforming growth factor-α (TNF-α). PTTG1 was constitutively overexpressed in cultured normal human keratinocytes (NHKs) via a retroviral vector, and the expression of TNF-α was analyzed. (**a**) Whole-cell lysates obtained from NHK cultures were subjected to immunoblotting analysis. NHKs overexpressing PTTG1 showed a thicker band compared with the control (CTR). The graphs indicate expression relative to β-actin (loading control) as determined by densitometry. (**b**) Total messenger RNA (mRNA) was extracted from NMK cultures, and the expression of TNF-α was analyzed by quantitative real-time PCR. NHKs overexpressing PTTG1 showed increased expression levels of TNF-α relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) compared with the control. (**c**) The concentration of TNF-α in conditioned medium obtained from the NHK culture was analyzed by ELISA. The concentration of TNF-α was increased in NHKs overexpressing PTTG1 compared with the control. All data except **c** are means ± SEM from at least three independent experiments. *$P<0.05$ vs. control; **$P<0.01$ vs. control.

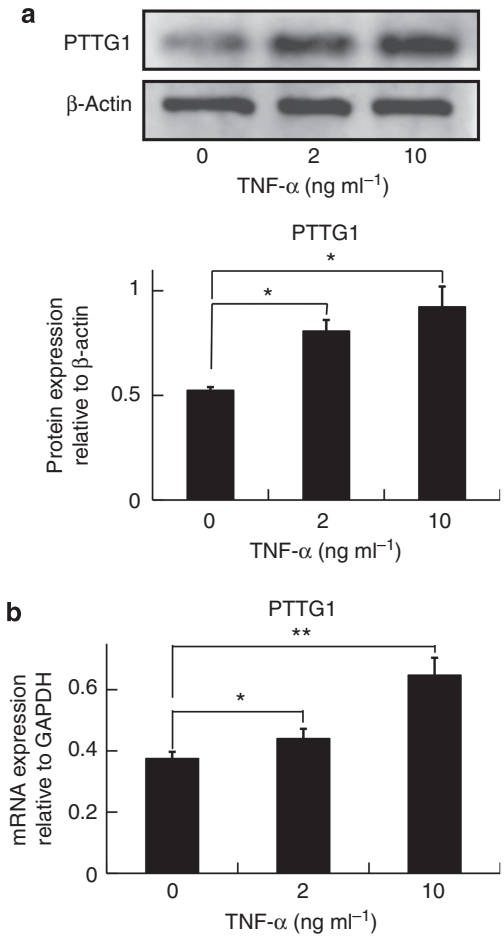

Figure 4. Pituitary tumor transforming gene 1 (PTTG1) expression is induced by transforming growth factor-α (TNF-α). Neonatal mouse keratinocytes (NMKs) were cultured with different doses of TNF-α (0, 2, 10 ng ml^{-1}) in growth medium, and the expression levels of PTTG1 were analyzed. (**a**) Whole-cell lysates obtained from NHK cultures were subjected to immunoblotting analysis. PTTG1 expression levels were increased in a TNF-α dose-dependent manner. The graph indicates the expression of PTTG1 relative to β-actin (loading control) as determined by densitometry. (**b**) Total messenger RNA (mRNA) was extracted from NMK cultures, and the expression of PTTG1 was analyzed by quantitative real-time PCR. The levels of PTTG1 expression relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were increased in a TNF-α dose-dependent manner. All data are means ± SEM from at least three independent experiments. *$P<0.05$ vs. control; **$P<0.01$ vs. control.

ligands appears to synergistically promote keratinocyte proliferation.

On the other hand, PTTG1 induced the production of TNF-α, and TNF-α itself had an inductive effect on PTTG1 expression in cultured NMKs. As PTTG1 had a positive effect on keratinocyte proliferation (Ishitsuka *et al.*, 2012), PTTG1 and TNF-α appear to have a synergistic effect on proliferation, and regulation of their expression may involve autoinduction, as seen in the EGFR ligands. However, there are some discrepancies regarding the effects of TNF-α on keratinocyte proliferation *in vitro*; several studies indicated a suppressive effect (Pillai *et al.*, 1989; Kono *et al.*, 1990; Banno *et al.*, 2004), whereas others showed an enhancing effect (Ziv *et al.*, 2008; Takahashi *et al.*, 2009). Indeed, although we attempted to confirm the positive effect of TNF-α on keratinocyte proliferation and the expression levels of PTTG1 in cultured NHKs, our results were inconclusive (data not shown). Although these discrepancies may have been due to differences in experimental settings, earlier studies using TNF-α (–/–) mice support our findings; the mice showed decreased proliferation capacity of keratinocytes in the early stages of chemical carcinogenesis, characterized by

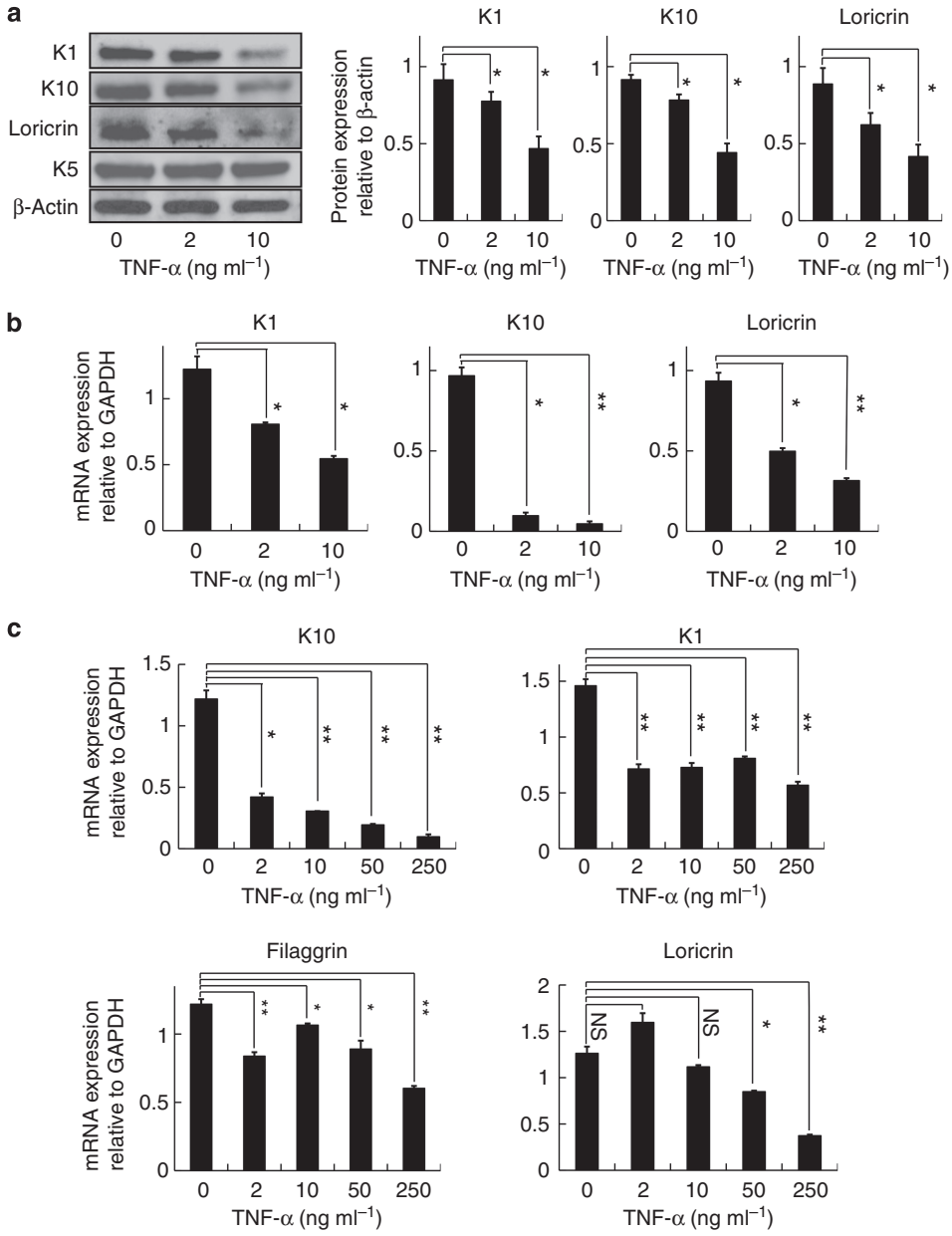

Figure 5. Transforming growth factor-α (TNF-α) suppresses keratinocyte differentiation. (**a**) Cultured neonatal mouse keratinocytes (NMKs) were differentiated by adding 0.35 mM Ca^{2+} to the medium containing different doses of TNF-α (0, 2, 10 ng ml^{-1}). Whole-cell lysates obtained from NMK cultures were subjected to immunoblotting analysis. The expression levels of K1, K10, and loricrin were decreased in a TNF-α dose-dependent manner. The graphs indicate their expression relative to β-actin (loading control) as determined by densitometry. (**b**) Total messenger RNA (mRNA) was extracted from NMK cultures, and the expression levels of the differentiation markers were analyzed by quantitative real-time PCR. The expression levels of K1, K10, and loricrin relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were decreased in a TNF-α dose-dependent manner. (**c**) Cultured NHKs were differentiated by adding 1.5 mM Ca^{2+} to the medium containing different doses of TNF-α (0, 2, 10, 50, 250 mg ml^{-1}). Total mRNA was extracted from NHK cultures, and the expression levels of the differentiation markers were analyzed by quantitative real-time PCR. The expression levels of K1, K10, filaggrin, and loricrin relative to GAPDH were decreased in the presence of TNF-α. In particular, the expression levels of K10 and loricrin relative to GAPDH were decreased in a TNF-α dose-dependent manner. All data are means ± SEM from three independent experiments. *$P<0.05$ vs. control; **$P<0.01$ vs. control. NS, not significant.

keratinocyte hyperproliferation and inflammation (Moore *et al.*, 1999; Suganuma *et al.*, 1999). In addition to keratinocyte proliferation, we also demonstrated a suppressive effect of TNF-α on early differentiation of keratinocytes, in addition to terminal differentiation, as reported previously (Kim *et al.*, 2011). As proliferation and differentiation are generally independent processes, the suppression of early differentiation may have been due to enhanced proliferation mediated by TNF-α; that is, TNF-α may enhance proliferation and suppress differentiation of keratinocytes, and PTTG1 may be involved in this regulatory mechanism by modulating the production of TNF-α.

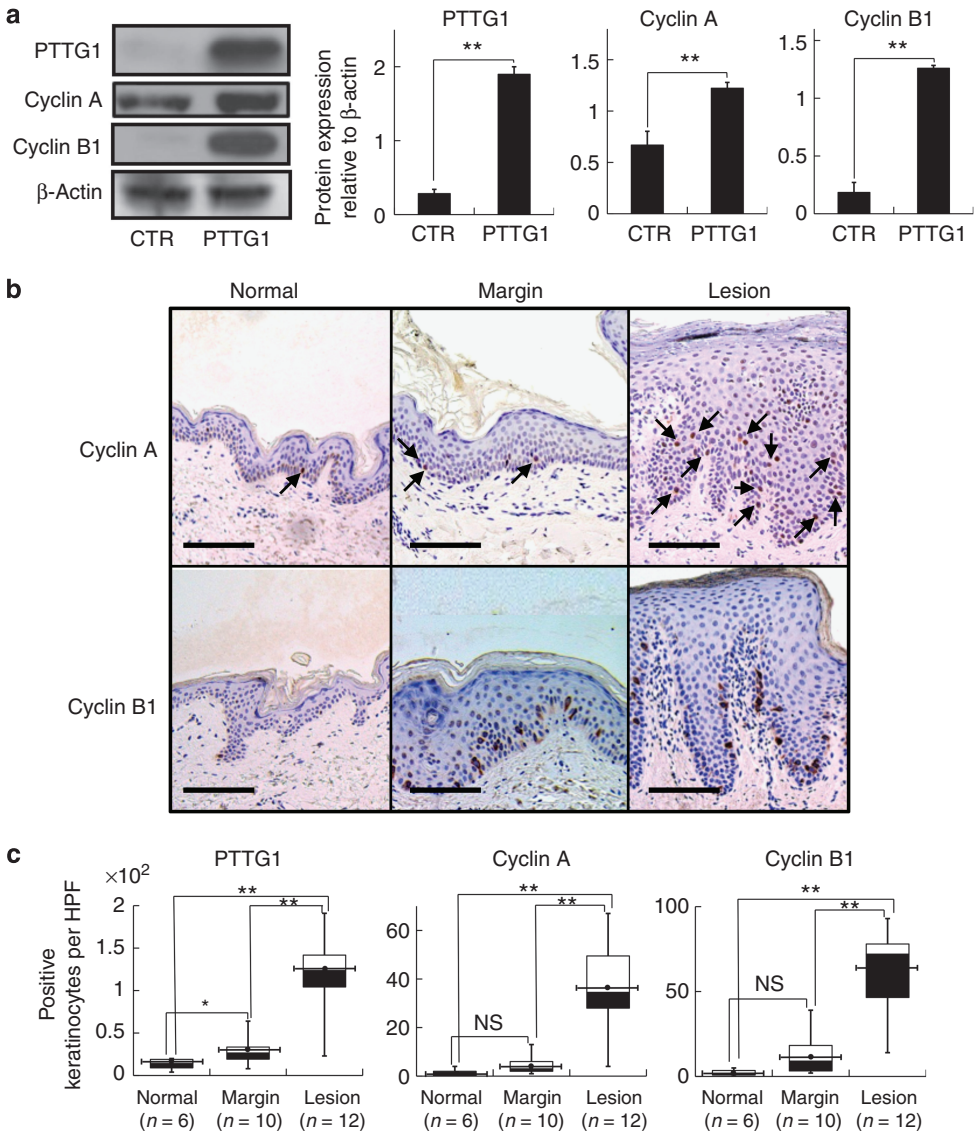

Figure 6. Pituitary tumor transforming gene 1 (PTTG1) overexpression in the psoriatic epidermis involves augmented expression of cyclin A and B1 proteins. The expression levels of cyclin A and B1 proteins were analyzed in cultured normal human keratinocytes (NHKs) and the samples obtained from patients with psoriasis and normal skin. (**a**) PTTG1 was overexpressed in NHKs, and the expression levels of PTTG1, cyclin A, and cyclin B1 were analyzed by immunoblotting. NHKs overexpressing PTTG1 showed the upregulation of cyclin A and B1 compared with the control (CTR). The graphs indicate their expression relative to β-actin (loading control) as determined by densitometry. (**b**) Sections of the samples obtained from patients with psoriasis (lesional area, $n=12$; marginal area, $n=10$) and normal controls ($n=6$) were subjected to immunohistochemical staining with antibodies against cyclin A and B1. Representative images are shown. Keratinocytes positive for cyclin A are indicated by arrowheads. In normal skin and marginal areas of psoriasis, cyclin A and B expression were mainly confirmed in the basal or peribasal keratinocytes, located in the cytoplasm and nucleus. The numbers of keratinocytes positive for cyclin A and B1 were increased in the lesional areas of psoriatic epidermis compared with normal skin or marginal areas. Black bars represent 100 mm. (**c**) The numbers of keratinocytes positive for PTTG1, cyclin A, and cyclin B1 per high-power field (HPF) were analyzed statistically in the samples. The numbers of keratinocytes positive for cyclin A and B1 in the lesional areas were significantly larger than those in the marginal areas or normal controls. However, there were no significant differences between the marginal areas and normal controls. The bottom of the black boxes and top of the white boxes indicate the lower and upper quartiles, respectively. The borders of the black and white boxes represent the medians. The whiskers with black dots crossing the boxes represent the means. The lower and upper whiskers represent the minimum and maximum values, respectively. *$P<0.05$ vs. control; **$P<0.01$ vs. control. NS, not significant.

We also demonstrated that PTTG1 in the psoriatic epidermis involves augmented expression of cyclin A and B1 proteins, the regulators of the cell cycle abundantly expressed in the S/G2 and G2/M phases of the cell cycle, respectively (Quereda and Malumbres, 2009). As PTTG1 protein is known to be abundant in these phases of the cell cycle (Ramos-Morales *et al.*, 2000), there may be some interactions between PTTG1 and the cyclin proteins, as suggested in earlier studies (Cohen-Fix and Koshland, 1999; Marangos and Carroll, 2008; Tinker-Kulberg and Morgan, 1999). Moreover, a previous study suggested that psoriasis is characterized by enhanced cyclin A and B protein expression in the epidermis (Miracco *et al.*,

2000). Taken together, PTTG1 overexpression in the epidermis seems to be closely related to abundant cyclin proteins and may be one of the characteristic features of psoriasis.

In conclusion, we demonstrated enhanced expression of PTTG1 in the psoriatic epidermis and its role in the pathophysiology of psoriasis characterized by aberrantly regulated keratinocyte proliferation/differentiation. Moreover, as a recent genetic study indicated that PTTG1 is a psoriasis susceptibility gene (Sun *et al.*, 2010; Chandran, 2012; Yang *et al.*, 2012), dysregulated expression of PTTG1 may contribute to disease progression. These observations suggested that PTTG1 is a key molecule and a potential therapeutic target of psoriasis.

MATERIALS AND METHODS

Immunofluorescence staining

Representative samples obtained from patients with psoriasis cut into sections at a thickness of 3 μm were deparaffinized and subjected to antigen retrieval and blocking with phosphate-buffered saline (PBS, pH 7.2) containing 10% goat serum, followed by overnight incubation with 1:1 mixtures of diluted primary antibodies (PTTG1, 10 μg ml^{-1}, ab-79546; ABcam, Cambridge, MA; Ki67, 1/25000, ab-86373; ABcam; K10, 10×, MON 3025; Sanbio B.V., Uden, The Netherlands; FLG, 2.5 μg ml^{-1}, MS-449; Thermo Fisher Scientific, Newark, DE). Antibody binding was visualized by incubation with a 1:1 mixture of secondary antibodies (anti-rabbit IgG-FITC, 2 mg ml^{-1}, sc-2012, and anti-mouse IgG-TR, sc-2781, 20 mg ml^{-1}; Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes at room temperature, followed by mounting with medium containing 4′6-diamidino-2-phenylindole (DAPI; VECTASHIELD mounting medium with DAPI, H-1200; Vector Laboratories, Burlingame, CA). A confocal microscopy system (TCS SP5; Leica Microsystems, Wetzlar, Germany) was used to observe and record the fluorescence.

Immunohistochemical staining

The deparaffinized sections were incubated overnight with primary antibodies diluted as follows after routine antigen retrieval and blocking: PTTG1, 10 μg ml^{-1}, ab-79546; ABcam; K10, 100×, MON3025; Sanbio B.V.; Ki67, 0.05 μg ml^{-1}, ab15580; ABcam; FLG, 2.5 mg ml^{-1}, MS-449; Thermo Fisher Scientific; cyclin A, 2 mg ml^{-1} RB-1548; Thermo Fisher Scientific; cyclin B1, 0.5 mg ml^{-1} 05-373; Millipore, Billerica, MA. After blocking of endogenous peroxidase with PBS containing 0.3% NaN_3 and 0.01% H_2O_2, visualization was performed using an EnVision system according to the standard protocol (Dako, Glostrup, Denmark), followed by light counterstaining with hematoxylin. PTTG1-, Ki67-, cyclin A–, and B1–positive keratinocytes were counted in high-power fields by two independent dermatologists (YI and YK) using a digital microscope (AX80; Olympus, Tokyo, Japan) and Flovel Filing System (Flovel, Tokyo, Japan), and the positivity rates for each immunogen were subjected to statistical analyses. According to the known intracellular locations, keratinocytes showing positivity in the cytoplasm and nucleus were counted as PTTG1-, cyclin A–, and cyclin B1–positive (Pines and Hunter, 1991Hagting *et al.*, 1999; Marangos and Carroll, 2008; Panguluri *et al.*, 2008), whereas nuclear staining was counted as Ki67 positivity (Gerdes *et al.*, 1984). Expression of K10 (1–4) was evaluated according to the numbers of K10-negative layers: Grade 1, all layers; Grade 2, 5–10 layers; Grade 3, 2–4 layers; Grade 4, one layer. Expression of FLG was evaluated according to the staining intensity scored on a scale from 0 to 4, with 0 indicating no staining and 4 indicating the most intense staining. Samples of marginal skin obtained from patients with nevocellular nevus ($n=6$) were used as normal controls.

Cell culture

Normal human neonatal or adult keratinocytes (NHKs) (KJB-100; DS Pharma, Osaka, Japan; FC-0007; Lifeline Cell Technology, Oceanside, CA) were cultured in serum-free growth medium (KJB-200; DS Pharma; LL-0007; Lifeline Cell Technology; 195130; Lonza, Basel, Switzerland), in accordance with the manufacturer's instructions. Constitutive overexpression of PTTG1 was obtained by transduction via retroviral vector as described previously (Fujisawa *et al.*, 2009; Ishitsuka *et al.*, 2012). NHKs and primary mouse keratinocytes prepared as described previously (Rothnagel *et al.*, 1993) were maintained at 80% confluency for 48 hours in growth medium containing 0.05 mM (NMK) or 0.06 mM (NHK) $CaCl_2$ with the indicated concentrations of human recombinant EGF (050-07141; Wako, Osaka, Japan), TGF-α (201-18341; Wako), and TNF-a (210-TA-010; R&D Systems, Minneapolis, MN) at 37 °C in 5% CO_2. NMKs were differentiated by maintaining it in medium containing 0.35 mM $CaCl_2$ and TNF-α at the indicated concentration for 48 hours following cultivation in EGF-free medium for 48 hours. Similarly, after depletion of EGF from the growth medium for 24 hours, NHKs were differentiated by maintaining them in medium containing 1.5 mM $CaCl_2$ and TNF-α at the indicated concentration for 96 hours with replacement of the medium at 48 hours. NHKs at third passage were used for the experiments.

Immunoblotting

Cells at 80–90% confluency in culture dishes were solubilized using sample buffer solution (Wako). The whole-cell lysates were subjected to SDS-PAGE on Mini-PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA), and the proteins were transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). The membranes were incubated overnight with primary antibodies against PTTG1 (10 μg ml^{-1}, ab-79546; ABcam), K10 (0.2 μg ml^{-1}, PRB-159P; Covance, Emeryville, CA), K1 (0.2 mg ml^{-1}, PRB-165P; Covance), keratin 5 (0.2 μg ml^{-1}, PRB-160P; Covance), loricrin (0.5 μg ml^{-1}, PRB-165P; Covance), TNF-α (13 μg ml^{-1}, GTX10205; GenTex, San Antonio, TX), cyclin A (10 μg ml^{-1} RB-1548; Thermo Fisher Scientific), cyclin B1 (0.5 μg ml^{-1} 05-373; Millipore), and β-actin (0.2 μg ml^{-1}; BioVision, Mountain View, CA), followed by incubation for 60 minutes with HRP-labeled secondary antibodies against rabbit IgG (0.04 μg ml^{-1}; Santa Cruz) or mouse IgG (0.4 μg ml^{-1}; Santa Cruz). Antibody binding was visualized and enhanced with SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific) and an image analysis system (LAS4000 mini; Fujifilm, Tokyo, Japan). The intensities of the bands were quantified using the Image J software (NIH, Bethesda, MD), and the expression levels were normalized relative to β-actin.

Quantitative RT–PCR analysis

Total RNA was extracted from culture dishes at 80–90% confluency using ISOGEN II (Nippon Gene, Tokyo, Japan), and cDNA was synthesized using a reagent kit (Prime Script RT reagent kit; Takara Bio, Shiga, Japan). A sequence detection system (ABI PRISM 7500;

Applied Biosystems, Foster City, CA) was used for SYBR Green (Takara Bio)–based quantitative analysis. The quantity of mRNA was normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The extracted RNA with OD_{260}/OD_{280} ratio > 1.8 was subjected to analysis, and quantification was performed in triplicate.

The primers used were as follows: TNF-α (human) (forward: 5′-TA TGGCCCAGACCCTCACA-3′; reverse: 5′- GGAGTAGACAAGGT ACAACCCATC-3′); PTTG1 (human) (forward: 5′- CCCTTGAGTG GAGTGCCTCT-3′; reverse: 5′- CACAGCAAACAGGTGGCAAT-3′); K1 (human) (forward: 5′-AGATCACTGCTGGCAGACATGG-3′; reverse: 5′-TGATGGACTGCTGCAAGTTGG-3′); K10 (human) (forward: 5′- CAACATCCTGCTTCAGATCGAC-3′; reverse: 5′- GCGCAGAGCT ACCTCATTCTCA-3′); FLG (human) (forward: 5′- CATGGCAGCT ATGGTAGTGCAGA-3′; reverse 5′-ACCAAACGCACTTGCTTTACA GA-3′); Loricrin (human) (forward: 5′- GGCTGCATCTAGTTC TGCTGTTTA-3′; reverse 5′- CAAATTTATTGACTGAGGCACTGG-3′); GAPDH (human) (forward: 5′- GCACCGTCAAGGCTGAGA AC-3′; reverse 5′- TGGTGAAGACGCCAGTGGA-3′); PTTG1 (mouse) (forward: 5′-TTTGGCATCTAAGGATGGGTTGA-3′; reverse: 5′-AGC ATTGAACACTTTGCCGACTC-3′); K1 (mouse) (forward: 5′-AGAAC ATGCAAGACCTGGTGGA-3′; reverse: 5′-TCTTGATGGTCACGA ACTCATTCTC-3′); K10 (mouse) (forward: 5′-CTGACAATGCCAA CGTGCTG-3′; reverse: 5′-GCAGGGTCACCTCATTCTCGTA-3′); Loricrin (mouse) (forward: 5′-GGTTGCAACGGAGACAACAGA-3′; reverse 5′-TTGGAAACCTCAGGCAAATTCA-3′); GAPDH (mouse) (forward: 5′-AAATGGTGAAGGTCGGTGTGAAC-3′; reverse 5′-CAA CAATCTCCACTTTGCACTG-3′).

ELISA

The conditioned media were obtained from NHKs overexpressing PTTG1 cultured under monolayer conditions. The concentrations of TNF-α were analyzed using an assay kit (EA-1041; Signosis, Sunnyvale, CA) in accordance with the manufacturer's protocol. The absorbance values were analyzed by fluorometry (Varioskan; Thermo Fisher Scientific) and given in arbitrary units relative to the values of the control for TNF-α.

Cell proliferation assay

Keratinocytes were grown in triplicate cultures in 96-well plates at a density of 5×10^4 per well (NMKs) or 1×10^4 per well (NHKs) in medium containing the indicated concentrations of EGFR ligands for 72 hours. NHKs overexpressing PTTG1 and the control cells were cultured in medium containing 2 ng ml^{-1} TGF-α. WST1 premix (MK 400; Takara Bio) was added to the culture in accordance with the manufacturer's protocol. The absorbance values at 440 nm were analyzed by fluorometry (Varioskan; Thermo Fisher Scientific).

Statistical analysis

Data are expressed as means ± SEM. Comparisons among groups were performed with the paired Student's *t*-test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Abou EL-Ela M, Nagui N, Mahgoub D *et al.* (2010) Expression of cyclin D1 and p16 in psoriasis before and after phototherapy. *Clin Exp Dermatol* 35:781–5

Aggarwal BB, Gupta SC, Kim JH (2012) Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. *Blood* 119:651–65

Banno T, Gazel A, Blumenberg M (2004) Effects of tumor necrosis factor-alpha (TNF-alpha) in epidermal keratinocytes revealed using global transcriptional profiling. *J Biol Chem* 279:32633–42

Carpenter G, Lembach KJ, Morrison MM *et al.* (1975) Characterization of the binding of 125-I-labeled epidermal growth factor to human fibroblasts. *J Biol Chem* 250:4297–304

Chandran V (2012) The genetics of psoriasis and psoriatic arthritis. *Clin Rev Allergy Immunol* 44:149–56

Clark RA, Kupper TS (2006) Misbehaving macrophages in the pathogenesis of psoriasis. *J Clin Invest* 116:2084–7

Cohen S (1962) Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J Biol Chem* 237:1555–62

Cohen-Fix O, Koshland D (1999) Pds1p of budding yeast has dual roles: inhibition of anaphase initiation and regulation of mitotic exit. *Genes Dev* 13:1950–9

Donetti E, Gualerzi A, Ricceri F *et al.* (2012) Etanercept restores a differentiated keratinocyte phenotype in psoriatic human skin: a morphological study. *Exp Dermatol* 21:549–51

Eckert RL, Adhikary G, Rorke EA *et al.* (2011) Polycomb group proteins are key regulators of keratinocyte function. *J Invest Dermatol* 131:295–301

Eckert RL, Crish JF, Banks EB *et al.* (1997) The epidermis: genes on - genes off. *J Invest Dermatol* 109:501–9

Ettehadi P, Greaves MW, Wallach D *et al.* (1994) Elevated tumour necrosis factor-alpha (TNF-alpha) biological activity in psoriatic skin lesions. *Clin Exp Immunol* 96:146–51

Fisher C, Haydock PV, Dale BA (1987) Localization of profilaggrin mRNA in newborn rat skin by in situ hybridization. *J Invest Dermatol* 88:661–4

Fujisawa Y, Nabekura T, Nakao T *et al.* (2009) The induction of tumor-specific CD4+ T cells via major histocompatibility complex class II is required to gain optimal anti-tumor immunity against B16 melanoma cell line in tumor immunotherapy using dendritic cells. *Exp Dermatol* 18:396–403

Gerdes J, Lemke H, Baisch H *et al.* (1984) Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 133:1710–5

Hagting A, Jackman M, Simpson K *et al.* (1999) Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. *Curr Biol* 9:680–9

Honeycutt KA, Koster MI, Roop DR (2004) Genes involved in stem cell fate decisions and commitment to differentiation play a role in skin disease. *J Investig Dermatol Symp Proc* 9:261–8

Ishitsuka Y, Kawachi Y, Taguchi S *et al.* (2012) Pituitary tumor-transforming gene 1 enhances proliferation and suppresses early differentiation of keratinocytes. *J Invest Dermatol* 132:1775–84

Kim BE, Howell MD, Guttman-Yassky E *et al.* (2011) TNF-alpha downregulates filaggrin and loricrin through c-Jun N-terminal kinase: role for TNF-alpha antagonists to improve skin barrier. *J Invest Dermatol* 131:1272–9

Kono T, Tanii T, Furukawa M *et al.* (1990) Effects of human recombinant tumor necrosis factor-alpha (TNF-alpha) on the proliferative potential of human keratinocytes cultured in serum-free medium. *J Dermatol* 17:409–13

Kristensen M, Chu CQ, Eedy DJ *et al.* (1993) Localization of tumour necrosis factor-alpha (TNF-alpha) and its receptors in normal and psoriatic skin: epidermal cells express the 55-kD but not the 75-kD TNF receptor. *Clin Exp Immunol* 94:354–62

Langley RG (2012) Effective and sustainable biologic treatment of psoriasis: what can we learn from new clinical data? *J Eur Acad Dermatol Venereol* 26(Suppl 2):21–9

Lowes MA, Chamian F, Abello MV *et al.* (2005) Increase in TNF-alpha and inducible nitric oxide synthase-expressing dendritic cells in psoriasis and

reduction with efalizumab (anti-CD11a). *Proc Natl Acad Sci USA* 102:19057–62

Marangos P, Carroll J (2008) Securin regulates entry into M-phase by modulating the stability of cyclin B. *Nat Cell Biol* 10:445–51

Mascia F, Mariani V, Girolomoni G *et al.* (2003) Blockade of the EGF receptor induces a deranged chemokine expression in keratinocytes leading to enhanced skin inflammation. *Am J Pathol* 163:303–12

Massague J (1983) Epidermal growth factor-like transforming growth factor. I. Isolation, chemical characterization, and potentiation by other transforming factors from feline sarcoma virus-transformed rat cells. *J Biol Chem* 258:13606–13

Miracco C, Pellegrino M, Flori ML *et al.* (2000) Cyclin D1, B and A expression and cell turnover in psoriatic skin lesions before and after cyclosporin treatment. *Br J Dermatol* 143:950–6

Moore RJ, Owens DM, Stamp G *et al.* (1999) Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. *Nat Med* 5:828–31

Nestle FO, Kaplan DH, Barker J (2009) Psoriasis. *N Engl J Med* 361:496–509

Nickoloff BJ, Karabin GD, Barker JN *et al.* (1991) Cellular localization of interleukin-8 and its inducer, tumor necrosis factor-alpha in psoriasis. *Am J Pathol* 138:129–40

Panguluri SK, Yeakel C, Kakar SS (2008) PTTG: an important target gene for ovarian cancer therapy. *J Ovarian Res* 1:6

Pei L, Melmed S (1997) Isolation and characterization of a pituitary tumor-transforming gene (PTTG). *Mol Endocrinol* 11:433–41

Pillai S, Bikle DD, Eessalu TE *et al.* (1989) Binding and biological effects of tumor necrosis factor alpha on cultured human neonatal foreskin keratinocytes. *J Clin Invest* 83:816–21

Pines J, Hunter T (1991) Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J Cell Biol* 115:1–17

Proksch E, Brandner JM, Jensen JM (2008) The skin: an indispensable barrier. *Exp Dermatol* 17:1063–72

Quereda V, Malumbres M (2009) Cell cycle control of pituitary development and disease. *J Mol Endocrinol* 42:75–86

Ramos-Morales F, Dominguez A, Romero F *et al.* (2000) Cell cycle regulated expression and phosphorylation of hpttg proto-oncogene product. *Oncogene* 19:403–9

Regnier M, Vaigot P, Darmon M *et al.* (1986) Onset of epidermal differentiation in rapidly proliferating basal keratinocytes. *J Invest Dermatol* 87:472–6

Roop DR, Hawley-Nelson P, Cheng CK *et al.* (1983) Keratin gene expression in mouse epidermis and cultured epidermal cells. *Proc Natl Acad Sci USA* 80:716–20

Rothnagel JA, Greenhalgh DA, Gagne TA *et al.* (1993) Identification of a calcium-inducible, epidermal-specific regulatory element in the 3′-flanking region of the human keratin 1 gene. *J Invest Dermatol* 101:506–13

Rothnagel JA, Mehrel T, Idler WW *et al.* (1987) The gene for mouse epidermal filaggrin precursor. Its partial characterization, expression, and sequence of a repeating filaggrin unit. *J Biol Chem* 262:15643–8

Santos-Briz A, Roncero M, Antunez P *et al.* (2009) Immunohistochemical expression of cyclin-dependent kinase-2 in psoriasis. *Br J Dermatol* 161:708–10

Suganuma M, Okabe S, Marino MW *et al.* (1999) Essential role of tumor necrosis factor alpha (TNF-alpha) in tumor promotion as revealed by TNF-alpha-deficient mice. *Cancer Res* 59:4516–8

Sun LD, Cheng H, Wang ZX *et al.* (2010) Association analyses identify six new psoriasis susceptibility loci in the Chinese population. *Nat Genet* 42:1005–9

Takahashi H, Tsuji H, Hashimoto Y *et al.* (2009) Cell proliferation and cytokine induction by tnf-alpha of psoriatic keratinocytes are not different from normal keratinocytes in vitro. *Indian J Dermatol* 54:237–9

Tinker-Kulberg RL, Morgan DO (1999) Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage. *Genes Dev* 13:1936–49

Tong Y, Eigler T (2009) Transcriptional targets for pituitary tumor-transforming gene-1. *J Mol Endocrinol* 43:179–85

Vlotides G, Cruz-Soto M, Rubinek T *et al.* (2006) Mechanisms for growth factor-induced pituitary tumor transforming gene-1 expression in pituitary folliculostellate TtT/GF cells. *Mol Endocrinol* 20:3321–35

Vlotides G, Siegel E, Donangelo I *et al.* (2008) Rat prolactinoma cell growth regulation by epidermal growth factor receptor ligands. *Cancer Res* 68:6377–86

Yang Q, Liu H, Qu L *et al.* (2012) Investigation of 20 non-HLA psoriasis susceptibility loci in Chinese patients with psoriatic arthritis and psoriasis vulgaris. *Br J Dermatol* 168:1060–5

Ziv E, Rotem C, Miodovnik M *et al.* (2008) Two modes of ERK activation by TNF in keratinocytes: different cellular outcomes and bi-directional modulation by vitamin D. *J Cell Biochem* 104:606–19

Zou H, McGarry TJ, Bernal T *et al.* (1999) Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science* 285:418–22